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Mapping polygenes for tuber resistance to late blight in a diploid *Solanum phureja* × *S. stenotomum* hybrid population

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With 2 figures

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Abstract

Potato tuber blight is a disease caused by the oomycete *Phytophthora infestans* (Mont.) de Bary. Due to the significant economic impact of this disease, introgression of durable resistance into the cultivated potato is one of the top priorities of breeding programmes worldwide. Though numerous resistance loci against this devastating disease have already been mapped, most of the detected loci are contributing towards foliar resistance while specific information on tuber resistance is limited. To identify the genetic components of tuber resistance and its relationship to foliar resistance and plant maturity we have investigated the host-pathogen interaction in a segregating diploid hybrid *Solanum phureja* × *S. stenotomum* family. Mature tubers from this mapping family were inoculated with a sporangial suspension of *P. infestans* (US-8 clonal lineage) and evaluated for lesion expansion. No significant correlation was detected between late blight resistance in foliage and tubers, and between plant maturity and tuber resistance. Four chromosomal regions were significantly associated with tuber resistance to the disease. The largest effect was detected near the marker locus *PSC* (LOD 10.7) located on chromosome 10. This locus explained about 63% of the total phenotypic variation of the trait. The other three resistance-related loci were mapped on chromosomes 8 (*GP1282*, LOD 4.4), 6 (*CP18*, LOD 4.0) and 2 (*CP157*, LOD 3.8). None of the four tuber resistance loci coincides with the foliage resistance loci detected in this same family. Tuber blight resistance quantitative trait loci (QTL) on chromosomes 2, 8 and 10 are distinct from the maturity QTLs and have an additive effect on tuber resistance. These results indicate that different genes are involved in foliar and tuber resistance to *P. infestans* in the present family and that some of the resistance genes might be associated with late maturity.

Key words: *Solanum* spec. — tuber blight — resistance — maturity — polygene mapping

Phytophthora infestans (Mont.) de Bary is an oomycete causing late blight disease in potatoes. All parts of the potato plant are susceptible to pathogen colonization. Severe epidemics can lead to massive destruction of foliage during the growing season and extensive tuber decay in storage. The current approach to managing tuber blight is to prevent foliar blight infection with application of fungicides, thus restricting access of spores to tubers in the ground. However, with the onset of metalaxyl-resistant isolates of *P. infestans* (Goodwin et al. 1996), the breeding effort to incorporate durable genetic resistance into new potato cultivars has intensified. Numerous genes for resistance to late blight have been recently described and their location on linkage maps was determined (for most recent reviews see Gebhardt and Valkonen 2001, Simko 2002). These genes are usually classified as either *R*-genes controlling race-specific (vertical) resistance, or polygenes controlling race-nonspecific (horizontal) resistance. Yet, the majority of currently identified genes are coding for foliar resistance, while very little is known about genes affecting resistance in tubers.

The relationship between late blight resistance in foliage and tuber can be ambiguous. Stewart et al. (1994) examined clones of five progenies where one parent had both foliar and tuber resistance and the other was susceptible. Foliar and tuber resistance were correlated, indicating either that both are determined by the same genes or by different, but closely linked genes. Similarly, Platt and Tai (1998) observed good correlations between foliar and tuber responses to the US-1 isolate of *P. infestans*. On the other hand, tuber resistance did not correlate with field foliar resistance when potato clones with various levels of resistance to US-8 isolate were evaluated by Douches et al. (2002). Foliar and tuber susceptibility to *P. infestans* were also not correlated in other tests involving the US-8 isolate, where low tuber susceptibility was associated with both extremely low and high foliar susceptibility (Kirk et al. 2001).

Molecular marker analysis of late blight-resistant loci also yielded contradictory results. Oberhagemann et al. (1999) and Collins et al. (1999) evaluated resistance in five hybrid families originating from crosses among seven different diploid potato clones. Although the major locus affecting tuber blight resistance was also the foremost locus involved in foliage blight resistance, the effects were inversed. Given the strong correlation with maturity at this locus, the authors hypothesized that the relative maturity of the tubers used to perform the tests might explain the outcome. At the time of testing, tubers from late maturing individuals were immature, when compared with those from the early maturing individuals, and therefore more susceptible to pathogen attack (Collins et al. 1999).

The objective of the present study was to identify the genetic components of tuber resistance and its relationship to foliar resistance and maturity in breeding material that has shown a relatively high level of resistance to late blight (Haynes and Christ 1999). We investigated the host–pathogen interaction on 125 clones originating from a diploid *Solanum phureja* × *S. stenotomum* hybrid family. This mapping population was previously evaluated for foliar blight resistance (Costanzo

et al. 2004, 2005) and plant maturity (Simko et al. 2004) and is considered devoid of *R*-genes. We also investigated if tuber resistance genes identified in this mapping population were previously detected in different genetic material.

Material and Methods

Plant materials: Two diploid clones (BD142-1 and BD172-1) were selected from the random-mated hybrid population of *S. phureja* × *S. stenotomum* that has shown relatively high levels of resistance to late blight in previous tests (Haynes and Christ 1999). The BD142-1 clone, which is highly susceptible to late blight under field conditions, was used as a female parent in a cross with the BD172-1 clone, which shows moderate resistance to *P. infestans* infection. The resulting 230 progeny (BD410 family) were evaluated for foliar late blight resistance in different years and locations (Costanzo et al. 2004). The average area under the disease progress curve score from previous trials was used in the present analysis. A subset of 132 randomly selected clones from this family were then used to construct a genetic linkage map using a total of 130 restriction fragment length polymorphism markers and two morphological traits (Costanzo et al. 2005). A slightly smaller subset of 130 individuals was screened for foliage maturity in a field experiment (Simko et al. 2004). The foliage maturity of plants was visually rated into five categories: (1) very late, (2) late, (3) intermediate, (4) early or (5) very early.

Disease assessment: Testing of tuber resistance to late blight was conducted on 125 clones from those used for the genetic linkage map construction. The level of resistance for an individual clone was evaluated in response to artificial inoculation with three *P. infestans* isolates representative of the US-8 clonal lineage. In previous assessments, these isolates had a compatible interaction (in combination) with potato plant differentials carrying *R*-genes 1, 2, 3, 4, 5, 6, 8, 9, 10 and 11 (Costanzo et al. 2004). To assess tuber blight resistance, 3-month-old tubers (counted from harvest date) were washed thoroughly with tap water, surface disinfected in a 5% sodium hypochlorite solution for 5 min and rinsed with distilled water. Each tuber was wounded on the surface (7 mm × 1 mm and 2 mm deep) with a sterilized tool and inoculated with a water suspension containing approximately 2000 sporangia (with approximate equal amounts from each isolate). The inoculated tubers were then placed, wounded side up, in plastic trays and incubated in the dark at 12°C and relative humidity of >90%. The experimental design consisted of six randomized complete blocks, with a single tuber per replication. After 12 days, the lesion size in mm was calculated from two measurements perpendicular to each other, and the overall average was used for statistical data analysis.

Data analysis: To detect loci associated with resistance to tuber blight we employed the maximum likelihood interval mapping method implemented in the computer program MapQTL version 4.0 (van Ooijen et al. 2002). A LOD score of 2.4, obtained by empirical permutation test with 1000 repeated shufflings (Churchill and Doerge 1994), was used as a threshold to declare the presence of a significant locus. The Multiple QTL Model (MQM) (Jansen 1993) feature of MapQTL was used for inclusion of background markers into the quantitative trait loci (QTL) analysis as cofactors. Because addition of the cofactors did not significantly affect the LOD score and the map location of detected loci, only results from the simple interval mapping are presented.

Results

No significant correlation ($r = 0.161$) was observed in the tested clones when comparing foliage and tuber resistance to the same isolates of *P. infestans* (Fig. 1). Similarly, the correlation between plant maturity and tuber resistance was

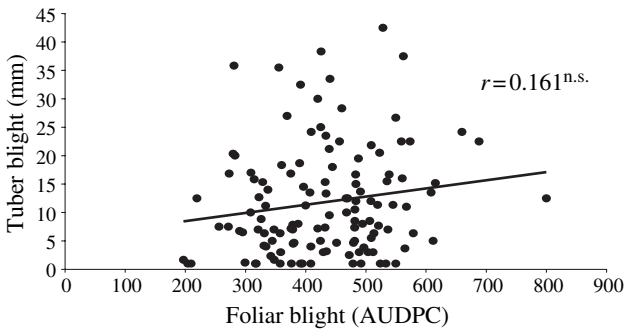

Fig. 1: Test of correlation between foliar and tuber resistance to late blight

not significant ($r = -0.109$). On the other hand, a strong correlation was found between plant maturity and foliage resistance to late blight ($r = -0.372$, $P < 0.0001$). In general, the more late maturing genotypes showed higher resistance to the disease.

Interval mapping analysis revealed that four chromosomal regions were significantly associated (LOD ≥ 2.4) with tuber blight resistance (Fig. 2). The largest effect was detected near the marker locus *PSC* (LOD 10.7) located on chromosome 10. This locus explained about 63% of the total phenotypic variation of the trait. The other three resistance-related loci were mapped on chromosomes 8 (*GP128*, LOD 4.4, 34%), 6 (*CP18*, LOD 4.0, 46%) and 2 (*CP157*, LOD 3.8, 20%). None of the four tuber resistance loci correspond to the foliage resistance loci previously detected on chromosomes 3, 5, 7 and 11 in this same BD410 family (Costanzo et al. 2005). To eliminate the possibility that tuber resistance is caused by an indirect effect of plant maturity, the relative location of QTLs for the two traits was compared. Despite low correlation between these two traits, three of four maturity-related QTLs were detected on the same chromosomes (6, 8, and 10) as

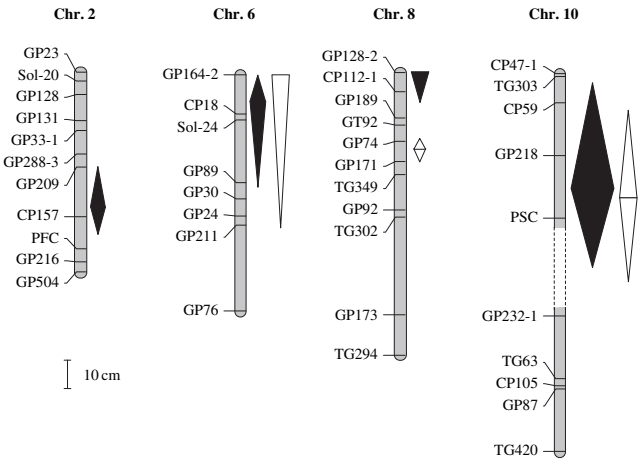

Fig. 2: Location of quantitative trait loci (QTLs) associated with tuber resistance to late blight. Relative size and the position of the resistance QTLs are indicated by black diamonds (triangle if at terminal marker). To give an impression of the magnitude, the length of a diamond illustrates chromosomal area significant at LOD ≥ 2.4, and the width of diamond corresponds to the maximum LOD score for the QTL. For comparison, maturity QTLs detected at the same chromosomes as tuber resistance loci are shown as white diamonds. White part of chromosome 10 indicates no significant linkage between markers

QTLs for the tuber blight resistance. However, the locus with the largest effect (LOD = 8.9) on plant maturity, residing on chromosome 5 near the marker *GP179*, is not linked with tuber resistance. Comparisons of the QTLs on chromosomes 6, 8 and 10 indicate that some of the observed resistance might be attributed to the indirect effect of plant maturity. On chromosome 6, the locations of QTLs for maturity and tuber resistance overlap and their LOD scores are similar (4.4 vs. 4.0). Moreover, the combination of alleles at this locus that leads to the earliest plant maturity is also associated with the highest susceptibility (data not shown). The situation on chromosome 8 is different and the two QTLs appear to be separated. The LOD score for tuber resistance is higher than the LOD score for plant maturity (4.4 and 2.9 respectively) and the combination of alleles contributes differently for the two traits. QTL locations for maturity and tuber resistance on chromosome 10 overlap. However, the resistance LOD score is substantially higher than the LOD score for maturity (10.7 vs. 4.4), and the combination of alleles associated with early plant maturity is related to both tuber resistance and susceptibility (data not shown). Therefore, we speculate that the resistance loci on chromosomes 8 and 10 do not correspond to the loci affecting plant maturity. To confirm this hypothesis linear regression analysis was used to partition tuber resistance into two separate components: one associated with plant maturity and the other associated with residual effect. Testing of the residual effect revealed a decline in the LOD score for tuber resistance on chromosome 6 from 4.0 to 2.6, but a small increase in the LOD score on chromosomes 2 (3.8–4.2), 8 (4.4–4.8) and 10 (10.7–10.8). These data, in conjunction with the effect of individual alleles, indicate that tuber blight resistance QTLs on chromosomes 2, 8 and 10 are distinct from maturity QTLs. However, the QTL on chromosome 6 is more likely to affect tuber resistance indirectly through plant maturity.

Further, we have examined the possible interaction among the three significant QTLs on chromosomes 2, 8 and 10. Three-way ANOVA confirmed significant effect of all three QTLs when they were analysed in a single model (*CP157*: $P = 0.009$; *GP128*: $P = 0.005$; *PSC*: $P < 0.0001$). Nevertheless, no significant interaction was detected among the three loci ($P = 0.264$), indicating their additive effect on tuber resistance. When favourable alleles were present at the three loci, the growth of mycelia was progressively inhibited from 17.6 ± 2.0 mm (no favourable allele) to 13.0 ± 1.4 mm (one favourable allele) to 8.0 ± 1.1 mm (two favourable alleles) to 5.6 ± 1.2 mm (three favourable alleles).

Discussion

Several studies have investigated the association between foliar and tuber resistance to late blight, but the results were often inconclusive. Platt and Tai (1998) observed similar foliar and tuber response to *P. infestans* infection when the same isolate was used for both inoculations. Stewart et al. (1994) concluded that foliar and tuber resistance are determined by the same (or closely linked) genes because of the correlation between the two traits in five tested progenies. However, the correlation between these traits was not confirmed in other studies, and low tuber susceptibility was associated both with extremely low and high foliar susceptibility (Kirk et al. 2001, Douches et al. 2002). Similarly, as in the later results, we did not detect a significant correlation ($r = 0.161$) between foliar and tuber resistance when using the US-8 isolate. Moreover, none of the four tuber-resistant loci from the BD410 family is located on the same chromosome as the loci associated with foliar resistance. In previous studies where populations devoid of *R*-genes were used for QTL mapping, a common major locus for foliar and tuber resistance was detected close to the marker locus *GP179* on chromosome 5 (Collins et al. 1999, Oberhagemann et al. 1999). However, its effect on foliar and tuber resistance was inversed. Oberhagemann et al. (1999) hypothesized that the different effect in the two tissues might be caused by a differential expression of multiple alleles and allele combinations. Given the presence of a strong, maturity-related locus in this chromosomal region (Collins et al. 1999, Oberhagemann et al. 1999, Simko 2002, Bradshaw et al. 2004), a more likely explanation is that both resistance traits are substantially affected by plant maturity (Collins et al. 1999). The effect of plant maturity on late blight resistance at this locus was also confirmed in a tetraploid mapping population (Bradshaw et al. 2004). Though analysis of the original values showed a strong effect of the locus on both foliar and tuber blight resistance, when the residuals from the regressions on maturity were analysed, no significant effect was observed (Bradshaw et al. 2004). Therefore, it appears that the QTL at this locus has a direct effect on maturity, which in turn affects the response to late blight.

The relationship between tuber and foliar resistance in populations carrying *R*-genes was examined recently in two studies. Mayton et al. (2005) tested two mapping populations (diploid and tetraploid) for resistance to tuber blight. The diploid mapping population originated from a cross between *S. tuberosum* and *S. berthaultii* and carried the *Rber* gene conferring resistance against late blight on foliage (Ewing et al. 2000). The tetraploid mapping population was produced from *S. tuberosum* cultivars and probably carried the *R1* resistance gene. When these populations were inoculated with incompatible isolates of late blight, segregation in resistance was observed, indicating involvement of the *R*-genes in tuber resistance. However, when clones from the two populations were inoculated with compatible isolates, only *Rber* provided residual resistance, while *R1* was not associated with tuber resistance (Mayton et al. 2005). Park et al. (2005) analysed tuber resistance in three mapping populations carrying *R*-genes or a major QTL for foliar resistance to late blight. In one mapping population, tuber blight resistance was inherited independently of foliar blight. In this population two *R*-genes (*R3a* and *Rpi-abpt*) function as foliage-specific *R*-genes, whereas the *R1* (or *R1*-like) gene acts on both the foliage and tuber. In the remaining two populations tuber and foliar blight resistance were significantly correlated, suggesting that both traits are conferred by the same gene (possibly *R3b*) and QTL respectively. One of the *R*-genes from the above studies – *Rber* – is located on chromosome 10 that carries a major QTL detected in the present study. The *Rber* gene comes from *S. berthaultii* (Ewing et al. 2000), while the QTL originates from *S. phureja* or *S. stenotomum* species. The position of these two resistance loci on the respective molecular maps is not identical; however, given the large confidence interval of QTLs (Simko 2002) and the somewhat different location of molecular markers in different mapping populations, we cannot exclude the possibility that the two loci are allelic versions of the same gene.

There is a well-documented relationship between plant maturity and foliage resistance to late blight (Visker et al. 2004, 2005, Jones and Simko 2005). Generally, the

early-maturing genotypes are more susceptible to *P. infestans* infection than the late-maturing genotypes, though some exceptions exist. However, the effect of plant maturity on tuber resistance response is more complex. Tubers of late-maturing genotypes might be resistant in laboratory assays performed on tubers from storage, but susceptible in the field because they are immature at harvest (Dorrance and Inglis 1998). If tubers at the time of testing are immature, and more susceptible to pathogen attack, late maturity will appear to be associated with higher susceptibility to disease (Collins et al. 1999, Oberhagemann et al. 1999). Conversely, a strong positive correlation between early maturity and susceptibility to tuber blight was observed in the tetraploid mapping population (Bradshaw et al. 2004). Furthermore, this strong correlation was confirmed on the individual loci level, when the allele associated with earlier maturity was also linked with higher susceptibility. In the present study, we did not detect any significant correlation between the two traits ($r = -0.109$) in the BD410 family. However, the allelic combination on chromosome 6 indicates that the early-maturing genotypes are more susceptible to the disease. As tubers used in the present experiment were stored for 3 months prior to inoculation, it is possible that the tuber aging process in storage also affected the maturity–resistance relationship. Dorrance and Inglis (1998) observed that resistance of tubers shortly after harvest is affected by periderm development, skin set or tuber maturity, while evaluation following storage examines the effects of tuber ageing.

Tuber resistance reaction to *P. infestans* infection appears to be a more complex process than previously thought. It has been observed that some regions of the potato genome are involved in both tuber blight and foliar blight resistance while other regions are specifically associated with one or the other (Collins et al. 1999, Oberhagemann et al. 1999, Bradshaw et al. 2004, Mayton et al. 2005, Park et al. 2005). Moreover, tests with multiple *P. infestans* isolates show that genotype × isolate interactions exist for all components of tuber blight resistance (Flier et al. 2001). Our results indicate that different genes are involved in foliar and tuber resistance to *P. infestans* in the diploid BD410 family, and that some of the resistance genes might be associated with late maturity. Hence, testing and selection for both resistance traits and maturity need to be carried out when introgressing late blight resistance loci from this germplasm into the cultivated potato.

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References

Bradshaw, J. E., B. Pande, G. J. Bryan, C. A. Hackett, K. McLean, H. E. Stewart, and R. Waugh, 2004: Interval mapping of quantitative trait loci for resistance to late blight [*Phytophthora infestans* (Mont.) de Bary], height and maturity in a tetraploid population of potato (*Solanum tuberosum* subsp. *tuberosum*). Genetics **168,** 983—995.

Churchill, G. A., and R. W. Doerge, 1994: Empirical threshold values for quantitative trait mapping. Genetics **138,** 963—971.

Collins, A., D. Milbourne, L. Ramsay, R. Meyer, C. Chatot-Balandras, P. Oberhagemann, W. De Jong, C. Gebhardt, E. Bonnel, and R. Waugh, 1999: QTL for field resistance to late blight in potato are strongly correlated with maturity and vigour. Mol. Breed. **5,** 387—398.

Costanzo, S., B. J. Christ, and K. G. Haynes, 2004: Late blight resistance in a diploid full-sib potato family. Plant Breeding **123,** 377—381.

Costanzo, S., I. Simko, B. J. Christ, and K. G. Haynes, 2005: QTL analysis of late blight resistance in a diploid potato family of *Solanum phureja* × *S. stenotomum*. Theor. Appl. Genet. **111,** 609—617.

Dorrance, A. E., and D. A. Inglis, 1998: Assessment of laboratory methods for evaluating potato tubers for resistance to late blight. Plant Dis. **82,** 442—446.

Douches, D. S., W. W. Kirk, M. A. Bertram, J. J. Coombs, and B. A. Niemira, 2002: Foliar and tuber assessment of late blight (*Phytophthora infestans* (Mont.) de Bary) reaction in cultivated potato (*Solanum tuberosum* L.). Potato Res. **45,** 215—224.

Ewing, E. E., I. Simko, C. D. Smart, M. W. Bonierbale, E. S. G. Mizubuti, G. D. May, and W. E. Fry, 2000: Genetic mapping from field tests of qualitative and quantitative resistance to *Phytophthora infestans* in a population derived from *Solanum tuberosum* and *Solanum berthaultii*. Mol. Breed. **6,** 25—36.

Flier, W. G., L. J. Turkensteen, G. B. M. van den Bosch, P. F. G. Vereijken, and A. Mulder, 2001: Differential interaction of *Phytophthora infestans* on tubers of potato cultivars with different levels of blight resistance. Plant Pathol. **50,** 292—301.

Gebhardt, C., and J. P. T. Valkonen, 2001: Organization of genes controlling disease resistance in the potato genome. Annu. Rev. Phytopathol. **39,** 79—102.

Goodwin, S. B., L. S. Sujkowski, and W. E. Fry, 1996: Widespread distribution and probable origin of resistance to metalaxyl in clonal genotypes of *Phytophthora infestans* in the United States and western Canada. Phytopathology **86,** 793—800.

Haynes, K. G., and B. J. Christ, 1999: Heritability of resistance to foliar late blight in a diploid hybrid potato population of *Solanum phureja* × *Solanum stenotomum*. Plant Breeding **118,** 431—434.

Jansen, R. C., 1993: Interval mapping of multiple quantitative trait loci. Genetics **135,** 205—211.

Jones, R. W., and I. Simko, 2005: Resistance to late blight and other fungi. In: M. K. Razdan, and A. K. Mattoo (eds), Genetic Improvement of Solanaceous Crops. Volume 1: Potato, 397—417. Science Publishers, Enfield, NH.

Kirk, W. W., K. J. Felcher, D. S. Douches, B. A. Niemira, and R. Hammerschmidt, 2001: Susceptibility of potato (*Solanum tuberosum* L.) foliage and tubers to the US8 genotype of *Phytophthora infestans*. Am. J. Potato Res. **78,** 319—322.

Mayton, H., W. De Jong, I. Simko, and W. E. Fry, 2005: Analysis of tuber blight resistance to *Phytophthora infestans*. 9th International Workshop on Plant Disease Epidemiology, Plant Disease Epidemiology: Facing 21st Century Challenges, Landerneau, France, April 11–15.

Oberhagemann, P., C. Chatot-Balandras, R. Schafer-Pregl, D. Wegener, C. Palomino, F. Salamini, E. Bonnel, and C. Gebhardt, 1999: A genetic analysis of quantitative resistance to late blight in potato: towards marker-assisted selection. Mol. Breed. **5,** 399—415.

van Ooijen, J. W., M. Boer, R. Jansen, and C. Maliepaard, 2002: MapQTL, Software for the Calculation of QTL Positions on Genetic Maps. Plant Research International, Wageningen.

Park, T. H., V. G. A. A. Vleeshouwers, J. B. Kim, R. C. B. Hutten, and R. G. F. Visser, 2005: Dissection of foliage and tuber late blight resistance in mapping populations of potato. Euphytica **143,** 75—83.

Platt, H. W., and G. Tai, 1998: Relationship between resistance to late blight in potato foliage and tubers of cultivars and breeding selections with different resistance levels. Am. J. Potato Res. **75,** 173—178.

Simko, I., 2002: Comparative analysis of quantitative trait loci for foliage resistance to *Phytophthora infestans* in tuber-bearing *Solanum* species. Am. J. Potato Res. **79,** 125—132.

Simko, I., K. G. Haynes, E. E. Ewing, S. Costanzo, B. J. Christ, and R. W. Jones, 2004: Mapping genes for resistance to *Verticillium albo-atrum* in tetraploid and diploid potato populations using haplotype association tests and genetic linkage analysis. Mol. Genet. Genomics **271,** 522—531.

Stewart, H. E., J. E. Bradshaw, and R. L. Wastie, 1994: Correlation between resistance to late blight in foliage and tubers in potato clones from parents of contrasting resistance. Potato Res. **37,** 429—434.

Visker, M. H. P. W., H. M. G. van Raaij, L. C. P. Keizer, P. C. Struik, and L. T. Colon, 2004: Correlation between late blight resistance and foliage maturity type in potato. Euphytica **137,** 311—323.

Visker, M. H. P. W., H. J. M. B. Heilersig, L. P. Kodde, W. E. Van de Weg, R. E. Voorrips, P. C. Struik, and L. T. Colon, 2005: Genetic linkage of QTLs for late blight resistance and foliage maturity type in six related potato progenies. Euphytica **143,** 189—199.